

Structural Modification for Antitumor Nitrogenous Steroid

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ABSTRACT Natural steroids have been showing notable cytotoxic activities, which are quite interesting lead compounds for the development of anticancer drug including estramustine and prednimustine. Considering that these semi-synthetic molecules are nitrogen mustard functionalized steroidal derivatives, the present review is focused on the methodologies of introducing nitrogen atom or nitrogen-containing heterocycles on A~D rings or side chains of steroids, and analysis of the structure-activity relationship (SAR) for these man-made cytotoxic steroids.

Keywords: steroids; structural modification; N-heterocycles; antitumor; structure-activity relationship;
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1 INTRODUCTION

Cancer is one of the leading causes of morbidity and mortality worldwide according to the WHO, and the incidence of most cancers including gastric cancer, liver cancer and esophageal cancer in our country has kept gradually increasing over the past 20 years from 1989 to 2008^[1]. Despite the availability of a large number of chemotherapeutic drugs, the medical need remains still largely unmet because of drawbacks of the antineoplastic agents, such as drug resistance, the lack of selectivity and cancer metastasis^[2].

Natural products are an important original source of many widely used anti-cancer drugs^[3]. As one of the widespread class of natural organic compounds occurring in plants, animals and fungi, steroid family has exhibited potent antitumor effects through direct suppression of tumor growth, including reduced cell cycle progression and apoptosis, or inhibiting tumor metastasis^[4,5], and numerous steroidal derivatives have been discovered with potent antineoplastic activity and high tissue selectivity^[6]. Cytotoxic steroids like estramustine phosphate, a hybrid structure of estradiol and nitrogen mustard combined through a carbamate

linkage with alkylating capability and estrogen-induced specificity, is marketed as a chemotherapy agent for the treatment of prostate cancer^[7, 8], which could also be used as a single chemotherapeutic agent or along with other agents such as 5α -reductase inhibitors^[9]. Due to the pivotal role of antineoplastic nitrogenous steroids, the present review will discuss the recent cases for the structural modification on the cyclic backbone (Fig. 1) and corresponding SAR for these steroidal derivatives as anticancer agents.

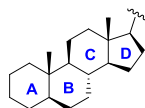


Fig. 1. Cyclic backbone of steroids

2 MODIFICATION OF A~D RINGS OF STEROID SKELETON

2.1 Modification on the A-ring

Poirier's group reported the preparation of five libraries of 2β -piperazino- 5α -androstane- $3\alpha,17\beta$ -diol derivatives by parallel solid-phase synthesis in 2011^[10], and biological evaluation against HL-60 leukemia cells found these aminosteroids revealed interesting SAR. For example, the amide coupling functionality showed stronger cytotoxic activity compared to the corresponding sulfonamides or benzylamines, and six of the most active amide derivatives (Fig. 2, **1~6**) were obtained with low half-maximal inhibitory concentration (IC_{50}) values of $1.7\sim 3.1\ \mu\text{M}$ and high selectivity indices of $5\sim 55$. In 2012, amino-pregnane derivatives (**7~9**) were further prepared by using a sequence of liquid and solid-phase reactions to extend the previous SAR data on anti-leukemic activities^[11], and derivative **9** substituted by the 3-acetylbenzoyl group exhibits strong anti-proliferation effect against HL-60 leukemia cells (IC_{50} : $1.9\ \mu\text{M}$) and low toxicity on normal peripheral blood lymphocytes (IC_{50} : $31\ \mu\text{M}$). Then, a 5α -androstane- $3\alpha,17\beta$ -diol derivative with a quinoline nucleus at the end of the piperazine-proline side-chain at position 2β and an ethynyl at position 17α (**10**)^[12, 13] showed the most potent cytotoxic activity against five cancer cell lines studied (IC_{50} : $0.1\sim 1.1\ \mu\text{M}$ for HL-60, MCF-7, T-47D, LNCaP and WEHI-3, respectively), and selectivity experiment towards liver enzymes CYP3A4 and CYP2D6 indicated the aminosteroid possessed a very low risk of drug-drug interactions. In order to increase the *in vivo* drug potency, an optimized 3-dimethylcarbamate aminosteroid derivative (**11**) was finally synthesized in 2016 according to the pro-drug strategy targeting on the 3α -hydroxyl group^[14], and the lead compound are more selective for cancer cells over normal cells and much more stable in liver microsomes.

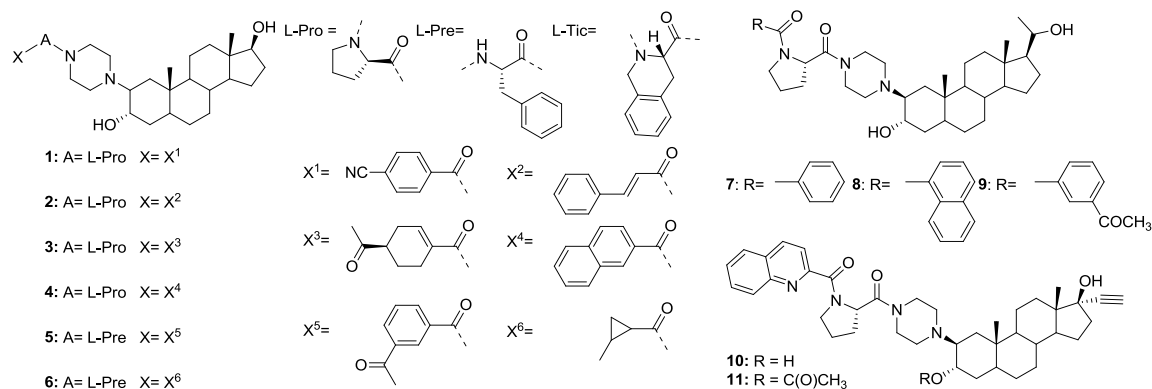


Fig. 2. Chemical structures of compounds 1~11

To develop more effective steroidal drugs, we synthesized a library of (25*R*)-2-[(1*H*-1,2,3-triazol-4-yl)methoxy]-spirostan-1,4,6-triene-3-ones from diosgenin for anti-tumor screening, and three steroidal triazoles (Fig. 3, **12~14**) possessed potent anti-proliferative effects against Caski cells with IC₅₀ of 9.4~11.8 μM^[15]. The structure/cytotoxicity investigations implied that the benzyl, 2-oxopropyl or 3-hydroxyphenylethyl substituted steroidal triazoles exhibited excellent anti-tumor activities.

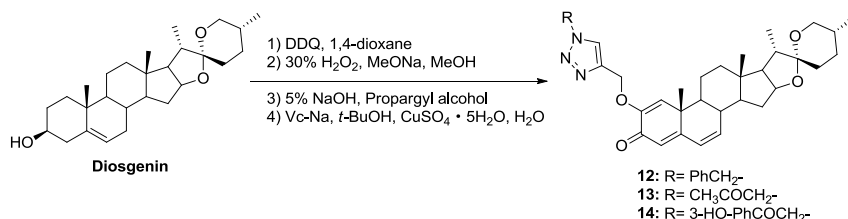


Fig. 3. Synthesis of compounds 12~14

Krstić^[16] reported the synthesis and anticancer activity of mono-/bis-thiosemicarbazones and mono-/bis-thiadiazolines substituted steroid at the C-3 position, and the best cytotoxic products were 3-thiosemicarbazones (Fig. 4, **15~17**) and 3,17-bis(thiadiazolines) (**18, 19**) against six cancer cell lines (HeLa, K562, MDA-MB-361, MDA-MB-453, LS174 and A549), which were comparable to cisplatin. The spiro heterocyclic substituent at the C-17 position, as well as the presence of an α,β -unsaturated thiosemicarbazone moiety at C-3, enhanced the activity of the tested compounds.

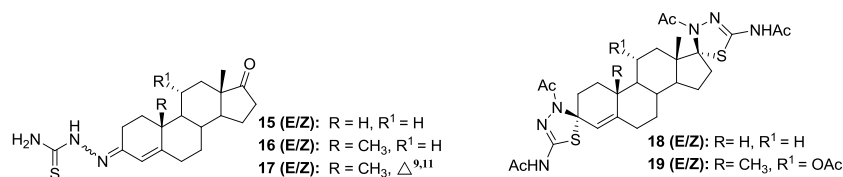


Fig. 4. Chemical structures of compounds 15~19

Bufadienolides are a type of natural cardiac steroids with potent antitumor activities from the Traditional Chinese medicine Chan'Su. In order to improve their biological activity and water solubility, Hu's group^[17] designed and synthesized the bufalin 3-nitrogen-containing-ester derivatives in 2013, and found the C3 moiety of A-ring had important influence on the cytotoxic activity in SAR study. The bufalin-3-piperidinyl-4-carboxylate (Fig. 5, **21**) displayed a significant cytotoxic potency compared to the parent compound bufalin with IC₅₀ values of 0.76 and 0.34 nM against HeLa and A549 cell lines, respectively. Several years later, they found bufalin-3-yl nitrogen-containing-carbamate hydrochloride (**22**, IC₅₀: 0.30 to 1.09 nM against ten tested tumor cell lines) and C4'-substituted oleandrin analogues (**23** and **24**, IC₅₀: 10.9 to 21.7 nM against human cervical carcinoma cell line) exhibited significant in vitro anti-proliferative activities^[18, 19].

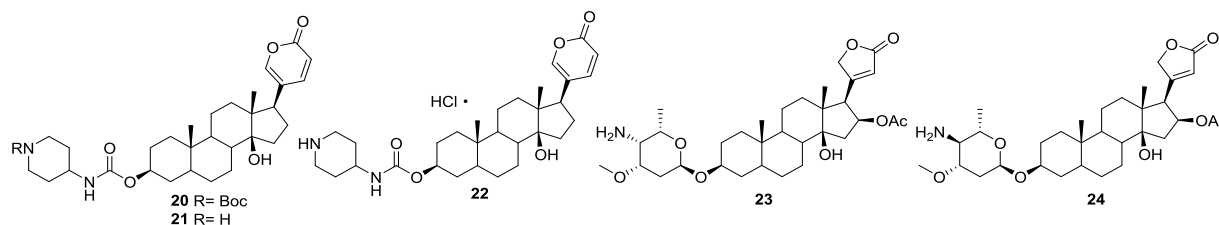


Fig. 5. Chemical structures of compounds 20~24

To clarify the SAR of anti-tumor activity of diosgenin derivatives *in vitro*, Fan's group^[20, 21] designed and synthesized 3-azole bromides substituted diosgenin derivatives (Fig. 6, **25** ~ **30**) based on the three-dimensional pharmacophore docking simulation for Bcl-2 inhibitors, and SAR results indicated that those with larger hydrophobic heterocyclic group have better activity through the mechanism of hydrogen bonding interaction and dipole-dipole interaction. Due to the restriction of molecular length, the side chain of compound **30** could not occupy the P2 and D3 sites compared with other derivatives, which therefore resulted in the poor antitumor activity.

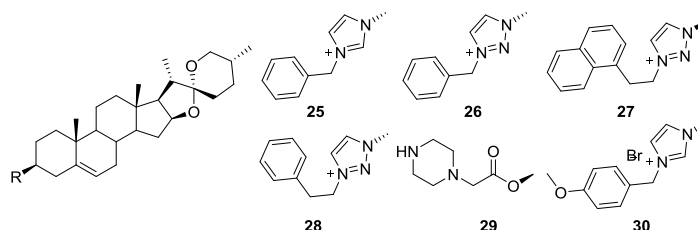


Fig. 6. Chemical structures of compounds 25~30

Duan's group firstly prepared E-salignone (Fig. 7, **31**)^[22] by eight steps of reactions in 16.3% total yields, and found it could inhibit the invasion of human breast cancer MDA-MB-231 cells and non-small cell lung cancer cells (A549) induced by the chemokine epidermal growth factor (EGF) with IC₅₀ values of 0.36 and 5.77 μ M, respectively. After modification, a series of antimetastatic E-salignone amide derivatives (**32**~**34**) were synthesized^[23], and compound **32** was found to possess anti-migration effects in wound-healing assay. Recently, they have further prepared pregn-17(20)-en-3-amine derivatives (**35**~**42**) as anti-breast cancer agents^[24]. Compared with the positive control LY294002 (IC₅₀: 0.38 μ M), the most potent anti-metastatic compound **40** exhibited 30 nM of IC₅₀ value through the mechanism of inhibiting the phosphorylations of integrin β 1, PI3K, Akt and PKC ζ . Preliminary SAR indicated 3 β -substituted steroid derivatives exhibited better anti-invasion activities than the 3 α -substituted ones, and the α,β -unsaturated fragment in ring D might be critical for their anti-metastatic activities.

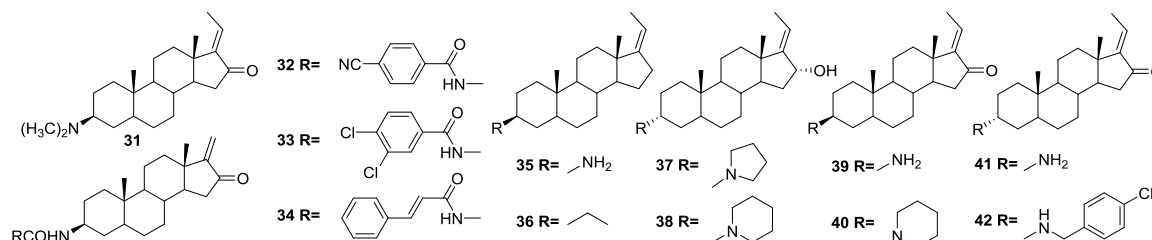


Fig. 7. Chemical structures of compounds **31**~**42**

Apart from the above acyclic chain nitrogen-containing derivatives on the A-ring of steroid skeleton, some heterocycle fused steroids were also reported as anticancer agents. Cephalostatin 1 (Fig. 8, **43**)^[25], a remarkably cytotoxic bis-steroidal pyrazine isolated from the marine tube worm *Cephalodiscus gilchristi*, has become an important target for total syntheses and structural modification model for the drug discovery. Tian's lab illustrated an efficient and practical synthesis of **43** using natural tetraol and lactone instead of the traditional progesterone or epiandrosterone^[26]. Pettit^[27] simplified its E and F rings by the replacement at C-17 with α -pyrone or dihydro- γ -pyrone, and two bis-steroidal pyrazine pyrones (**44**, **45**) were synthesized in 8 steps and found to have moderate anti-proliferative activities in the murine P388 lymphocytic leukemia cancer cell line with corresponding 50% effective dose (ED₅₀) of 25~57 μ M.

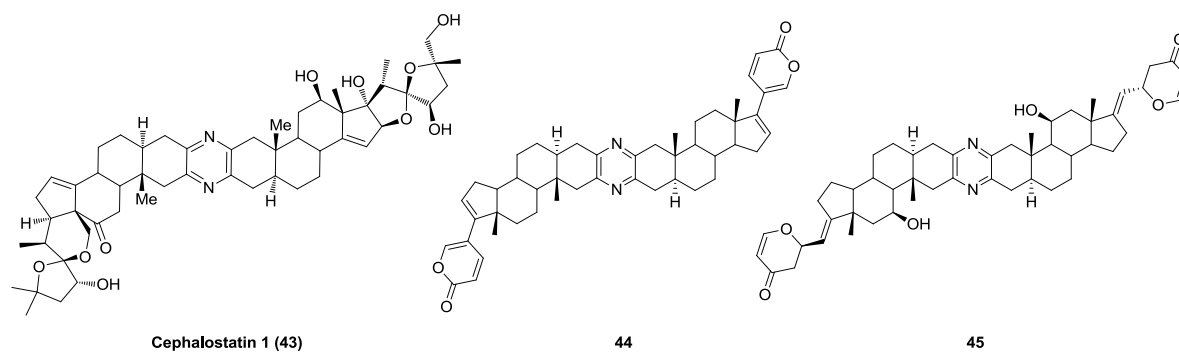


Fig. 8. Chemical structures of compounds 43~45

2.2 Modification on the B-ring

Due to the unique [6-6-6-5]-fused rings system, steroidal compounds display diverse biological activity and play a very important role in life, and a variety of steroidal drugs with unusual and interesting structures have been reported. With respect to the modification on steroid B-ring as anticancer agents, there are not many reported literatures.

Cui *et al.* prepared several series of substituted 5(6→7) abeo-sterols (Fig. 9, **46**, **47**)^[28] and 5(6→7) abeo-cholesterol derivatives (**48**~**51**)^[29], and investigated their anti-proliferative activity against some cancer cells. SAR studies indicated the presence of a cholesterol-type side chain was essential for the cytotoxicity, especially when a thiosemicarbazone group substituted at the C-6 position. Although the elimination of 5-hydroxyl has no obvious effect on their cytotoxic function, the removal of hydroxyl at the C-3 position decreased markedly the anti-proliferative activity of the compounds. The most potent analogue **48** exhibited excellent cytotoxic activities with an IC₅₀ value of 4.0 μM against Bel-7404 cells (compared with cisplatin, IC₅₀: 23 μM). The steroid *N*-methylthiazole derivatives (**52**, **53**) showed distinct anti-proliferative activity against A549 and HEPG2 cells, while the *N*-phenylthiazole substituted compounds (**54**, **55**) displayed a selective cytotoxic activity against HeLa cells and were almost inactive to HEK293T cells^[30]. They also studied the activity of steroidal semicarbazones (**56**~**58**) and thiosemicarbazones (**59**)^[31] and hydrazones, and found these compounds exhibited significant inhibitory activity against Bel-7404 cells with corresponding IC₅₀ values of 4.2~15.0 μM (cisplatin, IC₅₀: 11.6 μM). The steroidal *O*-benzyloxime ether with 6-thiosemicarbazone (**60**)^[32] displayed an excellent anti-proliferative activity against CNE-2 cancer cells owning an IC₅₀ value of 5.7 μM.

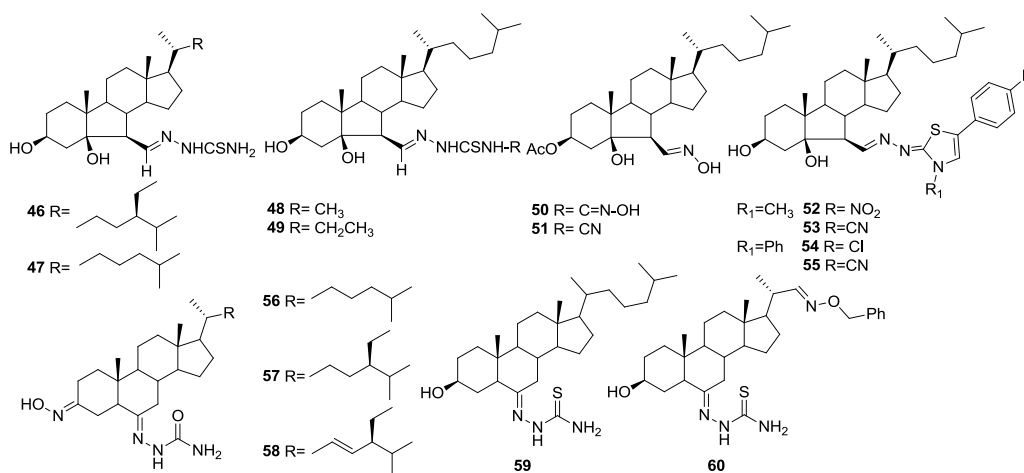


Fig. 9. Chemical structures of compounds 46~60

Steroidal 5,6-fused benzothiazines (Fig. 10, **61~63**) were also reported by Shamsuzzaman as cancer chemotherapeutic agents^[33, 34] from cholest-5-en-7-one derivatives in high yield, and their molecular docking studies with DNA duplex of sequence d(CGCGAATTCGCG)2 dodecamer (PDB ID: 1BNA) indicated the electrostatic and hydrophobic interactions between nucleotide base pairs and the amino group in the compounds were the main contribution for the high activity.

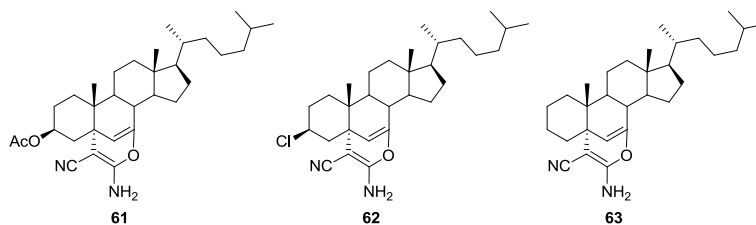


Fig. 10. Chemical structures and docking simulations for compounds 61~63

2.3 Modification on the C-ring

The introduction of nitrogen atom in steroidal molecule by a heteroatom affects the chemical properties and often resulted in the alteration of its biological activities, such as lowering the acute toxicity, enhancing the inhibition of enzyme, or improving the antitumour activity^[35, 36].

Hanson used different lengths of triazole linker to connect 11 β -(4-substituted oxyphenyl)estradiols and geldanamycin component, and found the final antiestrogen-geldanamycin conjugates (Fig. 11, **64, 65**)^[37] retained significant anti-proliferative activity against two breast cancer cell lines with respective IC₅₀ values below 102 nM. These results indicated further modifications in both ER-targeting strategies and linking groups were needed in order to achieve greater potency and selectivity in therapeutic drug delivery.

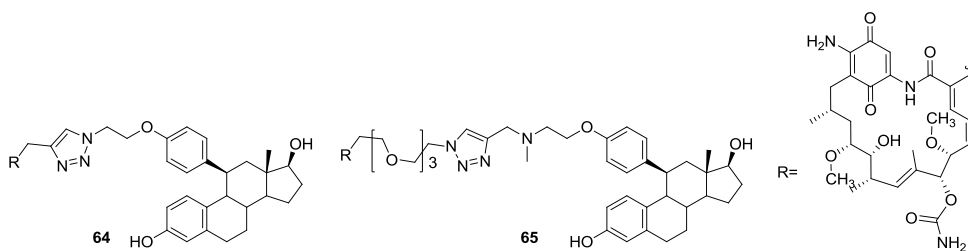


Fig. 11. Chemical structures of compounds 64~65

Metapristone (Fig. 12, **67**) was the major active metabolite of mifepristone (**66**), which was confirmed by Shao and co-workers^[38] as a good cancer metastatic chemopreventive agent with modest cytostatic effects including cell cycle arrest, mitochondrial membrane potential, and apoptosis on human colorectal cancer HT-29 cells. The computational docking provided the evidence that the two drugs exert their pharmacological effects with key hydrogen-bonding contacts in the same active center of hydrophobic binding cavity composed by Gln570, Arg611 and Gln642. The *N*-demethylation condition for large-scale preparation of **66** was improved in a significantly higher yield (97%) by using suitable base LiOAc according to Chen's method^[39], and gram scale of the product could be synthesized for its further clinical trials.

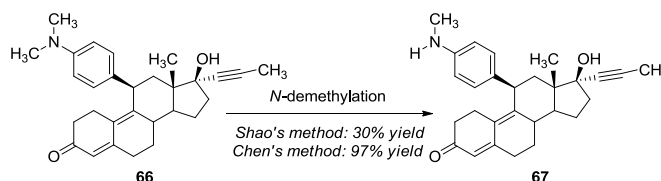


Fig. 12. Chemical structures of compounds 66~67

Most breast cancers are initially hormone-dependent diseases, so structural modifications of steroidal hormones also lead to steroidal inhibitors targeting on aromatase or steroid sulfatase for the treatment of estrogen-dependent (ER+) breast cancers^[40]. Mernyak^[41] found D-secooximes in the 13 β - and 13 α -estrone series (Fig. 13, **68~70**) displayed high in vitro anti-proliferative potential against a number of cancer cell lines, with IC₅₀ values in a low submicromolar range. Recently, several *p*-alkylbenzyl-substituted triazoles in the 13 β and 13 α -D-secoestrone series (**71~74**)^[42] have been synthesized and found to selectively exert high cytostatic action against A2780 cells with IC₅₀ values of 1 μ M, and these secosteroid triazoles could effectively suppress the estrone to 17 β -estradiol conversion with IC₅₀ values in low micromolar range through inhibiting the activity of human 17 β -hydroxysteroid dehydrogenase type 1.

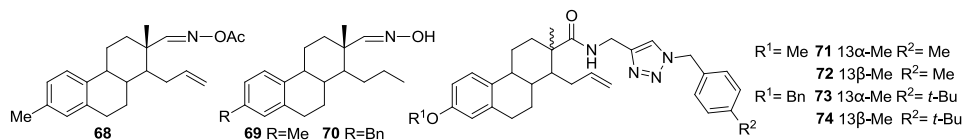


Fig. 13. Chemical structures of compounds 68~74

2.4 Modification on the D-ring

The investigation on the SAR of D-ring modification on steroidal skeleton was also attractive for medicinal chemists. Being lack of proper directing groups, the substitution (e.g., the introduction of an azido group) on steroidal D-ring was always proved to be more difficult^[43].

Zupko and co-workers^[44] reported the stereo-selective synthesis of 15β -triazolyl- 5α -androstanes (Fig. 14, **75~78**) from 1α -azidoandrostanes, which showed noteworthy *in vitro* cytostatic effects against HeLa, MCF 7 and A431 cell lines with corresponding IC_{50} of $1.69\sim 8.40\ \mu\text{M}$. Yu's group^[45] synthesized the steroidal spiro-pyrrolidinyl oxindoles (**79, 80**) with good anti-proliferative activities (IC_{50} : $0.71\sim 4.33\ \mu\text{M}$ against SMMC-7721 and MCF-7, respectively), which exerted anticancer mechanism by inducing cellular early apoptosis and arresting cell cycle at G2/M phase in a concentration- and time-dependent manner.

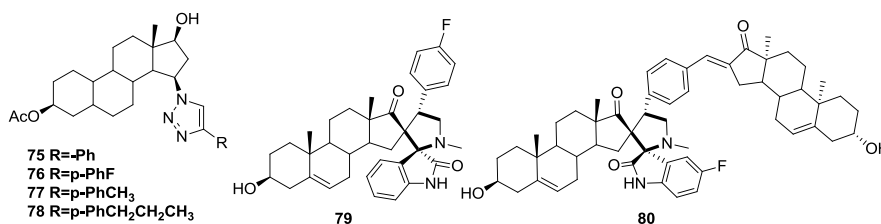


Fig. 14. Chemical structures of compounds 75~80

The trans-16-triazolyl-13 α -methyl-17-estradiol derivatives (Fig. 15, **81~84**) were prepared by Mernyak in 2015^[46, 47], and the SAR for stereo-configurations of various substituent at C-16 and C-17 of steroidal was also studied. The $16\beta, 17\alpha$ isomers are generally proved to be potent on all the cancer cell lines tested, which are usually substituted by *p*-alkyl substituents on the triazolyl-phenyl and displayed outstanding activities.

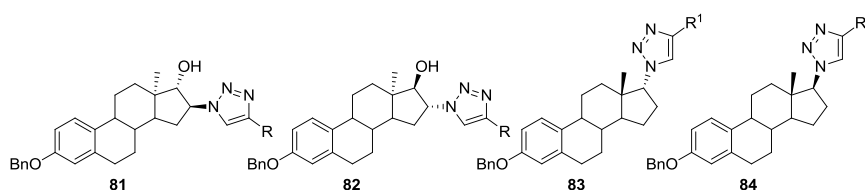


Fig. 15. Chemical structures of compounds 81~84

With the aim of studying the SAR of novel dehydroepiandrosterone derivatives containing triazole or pyrazole rings at C-17, Cabeza^[48] prepared these heterocycles substituted steroid (Fig. 16, **85**~**87**) by multi-step organic reaction in high yields. Biological evaluation indicated the triazole ring derived at C-17 of steroid showed much higher cytotoxic activity as compared to a pyrazole substituted at the same position, which might be attributed to higher number of hydrogen bond acceptors of nitrogen atoms in triazole systems. After that, Banday reported a series of novel D-ring substituted isoxazoline and oxazoline derivatives of dehydroepiandrosterone and pregnenolone (**88**~**91**)^[49] as potential anti-prostate cancer agents. He and co-workers^[50] also designed and synthesized 17-(2',5'-disubstituted-oxazolyl)-androsta-4,16-dien-3-one derivatives via the gold-catalyzed steroidal alkyne oxidation from 4-androstene-3,17-dione (**92**~**95**), and most of them exhibited potent antitumor activities with IC₅₀ of 3.0~25.5 μmol/L against MCF-7 cell line. To further study the SAR of steroidal pyrazole analogs, Shi and co-workers^[51] modified 17β-pyrazolyl steroid derivatives through a concise way from pregnenolone. Compound **96** exhibited excellent cytotoxicity against A549 with an IC₅₀ value of 0.91 μM, which implied 4-chlorophenyl and NH groups were important for better anti-proliferative effects.

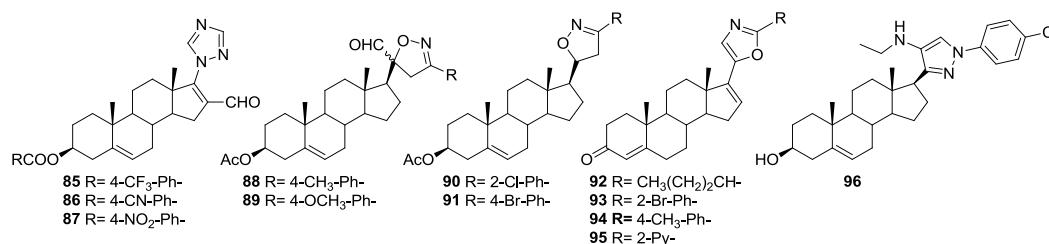


Fig. 16. Chemical structures of compounds **85**~**96**

In order to study the anticancer effect of the D-ring fused steroidal heterocycles, several pyridazino-, pyrimido-, quinazolo-, oxirano- and thiazolo-steroid derivatives were synthesized in Elmegeed's group^[52]. Both acetonitrilothiazolyl androstane derivative (Fig. 17, **97**) and its copper complex (**98**) exhibited more inhibitory influence on the MCF-7 growth than the reference drug doxorubicin (Dox) after 48 h incubation. Liu and coworkers^[53] also prepared a series of steroidal[17,16-d]thiazole, steroidal[1,2-b]pyridine and steroidal[17,16-d]thiazole[2,1-b]imidazo products through a convenient and productive method, and found imine-substituted steroidal[17,16-d]thiazole (**99**) had a potent cytotoxicity against MGC-803 with the IC₅₀ value of 3.75 μM. The majority of amino-substituted steroidal[17,16-d]thiazole showed relatively better activity against the three cell lines, and the most highly active compound was **100** which strongly inhibited the proliferation of EC109 cells with the IC₅₀ value to be 0.196 μM.

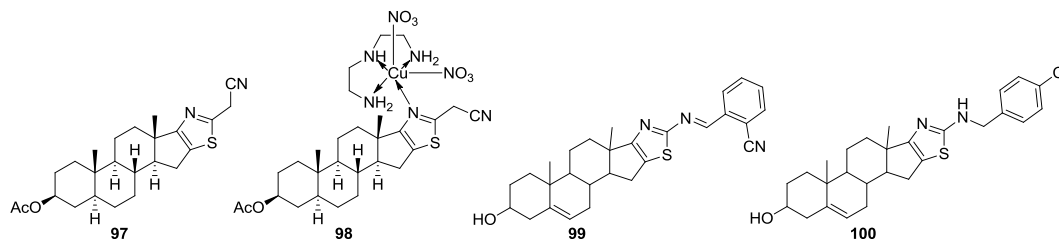


Fig. 17. Chemical structures of compounds 97~100

2.5 Other modifications

E/F-Spiroacetal ring modified steroids were identified as a useful template for the design of antitumor agents and a challenging subject for the development of novel synthetic methodologies. In 2014, Negi^[54] reported the synthesis of furostane derivatives from diosgenin through opening the F-spiroacetal ring by using sodium cyanoborohydride in AcOH at room temperature, and the final steroidal aldoxime (Fig. 18, **101**) and oxime acetate (**102**) derivatives could strongly inhibit the proliferation of five cancer cell lines like tamoxifen. Wang's group^[55] synthesized a range of aza-sapogenin derivatives by a facile synthetic route from natural sarsasapogenin, and the pharmacological results showed that most of the products displayed excellent selective cytotoxicity toward the cancer cell lines. Compared with the activities from a bromo or morpholinyl substituent at the C-3 and C-26 positions of sapogenin, the C-3/C-26 amino derivatives generated much better cytotoxic effects, and compound **103** (IC₅₀: 0.56 μ M against A375-S2 cells; 0.72 μ M against HT1080 cells) and **104** (IC₅₀: 1.70 μ M against A549 cells) exhibited significantly cytotoxic activity.

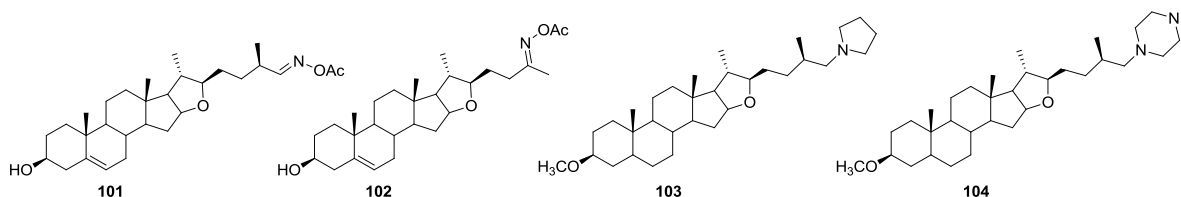


Fig. 18. Chemical structures of compounds 101~104

Using diosgenin as the starting material, Fan's group^[20, 21] also designed to synthesize a series of cytotoxic F-spiroacetal ring-opening products substituted by imidazole hydrobromide (Fig. 19, **105~108**) with IC₅₀ values of 2.99~8.94 μ M against K562 or A549 cells. After further modification, A- and F-ring opening steroid (**109**)^[56] was found to possess similar pharmacological activity with Taxol, which indicated introducing more bulky hydrophilic groups on A- and D-rings might be beneficial for antitumor effect.

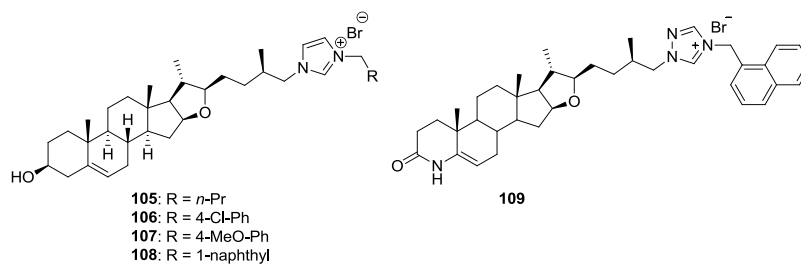


Fig. 19. Chemical structures of compounds 105~109

Barrera's group^[57] synthesized two pregnane derivatives with a triazole (Fig. 20, **110**) or imidazole (**111**) moieties at the C-21 position using commercial 16-dehydropregnenolone acetate as starting materials, and the two compounds could inhibit the proliferation of prostate cancer (PC-3), breast cancer (MCF7) and lung cancer (SK-LU-1) cell lines in a dose-dependent manner. **111** had an antagonist effect upon progesterone receptor and discarded completely the androgen receptor or vitamin D receptor pathways as possible action mechanism upon anti-proliferative process, suggesting the modification of steroidal structure by introducing imidazole and triazole moieties deserve further investigation.

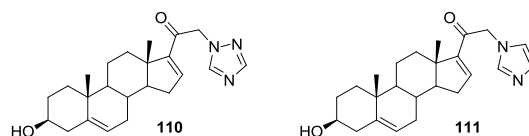


Fig. 20. Chemical structures of compounds 110 and 111

Cui's group^[58, 59] reported a series of dehydroepiandrosterone-17-hydrazone derivatives possessing various aromatic heterocycle structures in 17-side chain of steroidal nucleus (Fig. 21, **112**~**114**), which showed distinct anti-proliferative activity against some cancer cells *in vitro* through inducing cancer cell apoptosis mechanism. Compared with **112** containing a quinoline structure (IC₅₀: 1.0 μM in SGC 7901 cells), the indole substituted product **113** showed a selective cytotoxicity against HeLa cells with the IC₅₀ value of 5.0 μM.

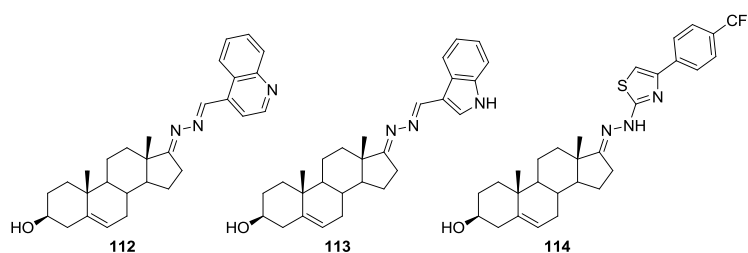


Fig. 21. Chemical structures of compounds 112~114

3 CONCLUSION

Although there are many chemotherapy drugs currently on the market, the consequent side effects and tumor metastasis seriously affect the drug efficiency^[2]. Recent research work indicated that more than 60% of the current anticancer chemotherapeutic drugs used in clinic were initially developed from natural products^[2, 60]. Steroids are abundant in nature with diversity of structures and wide bioactivities, which are widely used as antitumor agents for a long period of time, such as the phytosterols^[4, 61], diosgenin^[62] and cardiotonic steroids^[63]. The chemical modification of some natural steroids has been proven a more effective way for the development of anticancer agents, and lots of lead compounds including OSW-1^[64] and 2-methoxyestradiol^[65] were found as potential therapeutic agents for cancer therapy, which also indicated introducing heteroatoms like oxygen and nitrogen really altered the chemical and biological properties of a steroid. Therefore, the potential development of heterosteroid as anticancer agents, especially for azasteroids^[6, 35], has gained increasing attention in medicinal field due to the unique heteroatom effects^[66].

In this review, some recent progresses on the introduction of anticancer bioactive pharmacophores to steroid motifs with the aim of improving the drug efficiency and selectivity were summarized, and the synthetic method and SAR studies for nitrogen mustard hybrids, *N*-heterocycles substituted or fused steroids were reported. The classic nitrogenous chain (such as amide, imine, oxime, hydrozone, *etc.*) and 5/6-membered nitrogen heterocycles were proved as effective pharmacophores to produce better antitumor effect for steroids. SAR investigations also indicated the reason why these nitrogenous steroids exhibited better biological activity than their precursors was attributed to the bulky stereo-configuration, aromaticity, or the possibilities to form hydrogen bonds with biological macromolecules. Hopefully, this review could provide reference and suggestions for the scientists and medicinal chemists for the successful development of nitrogenous steroidal derivatives as novel antitumor drugs.

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Structural Modification for Antitumor Nitrogenous Steroids

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The methods for introducing nitrogen-containing chains or N-heterocycles on A~D rings or the side chains of steroids were summarized in this review, and the structure-activity relationship (SAR) for these nitrogenous steroids was also discussed.

